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IRON IN MULTIPLE MYELOMA

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Abstract

Multiple myeloma is a non-curable B cell malignancy in which iron metabolism plays an important role. Patients with this disorder almost universally suffer from a clinically significant anemia, which is often symptomatic, and which is due to impaired iron utilization. Recent studies indicate that the proximal cause of dysregulated iron metabolism and anemia in these patients is cytokine-induced upregulation of hepcidin expression. Malignant myeloma cells are dependent on an increased influx of iron and therapeutic efforts are being made to target this requirement. The studies detailing the characteristics and biochemical abnormalities in iron metabolism causing anemia and the initial attempts to target iron therapeutically are described in this review.

Keywords

multiple myeloma; hepcidin; interleukin-6; bone morphogenetic protein; transferrin receptors

I. INTRODUCTION

Iron metabolism is significantly altered in multiple myeloma (MM). Availability of iron for the developing erythron becomes limiting resulting in the characteristic anemia so frequently seen in this disease. In contrast, the availability of iron for the expanding malignant clone remains a critical determinant of MM progression. This review will focus on these two aspects of iron metabolism. First, the role of hepcidin in the dysregulation of iron balance and resulting anemia will be considered. We will then briefly describe early therapeutic attempts to specifically deprive the malignant plasma cell of iron.

II. ROLE OF HEPcidIN AND DYsREGULATED IRON METABOLISM IN ANEMIA OF MULTIPLE MYELOMA

A. Anemia in Multiple Myeloma

Multiple Myeloma is a clonal disorder of plasma cells that accounts for approximately one percent of all cancers and ten percent of hematologic malignancies.¹ It is a disease of the elderly with a median age at diagnosis of 66 years and less than ten percent of cases occurring in patients younger than 50 years.¹ Although new therapeutics have improved the prognosis, myeloma remains incurable with a median survival of 7–8 yrs.² It is 2–3 times more common in African Americans than in whites and is slightly more common in men than women.^{1,3} Multiple myeloma is known to arise from a precursor pre-malignant condition known as Monoclonal Gammopathy of Undetermined Significance, or MGUS. MGUS carries a risk of progression to multiple myeloma of 1 percent per year.⁴ Accordingly, patients with MGUS are typically monitored expectantly for progression.

Anemia is a frequent finding in myeloma patients. One large retrospective analysis of more than one thousand patients found that 73% of myeloma patients were anemic at the time of diagnosis with hemoglobin levels <12g/dL.⁵ Other studies^{6,7} have demonstrated that more than 95% of myeloma patients will suffer from anemia at some point during their disease. Anemia is typically moderate with hemoglobin concentrations between 8 and 10 g/dL in most patients.^{5–7} However, up to 10% of myeloma patients have hemoglobin concentrations less than 8g/dL and, in general, anemia impacts quality of life⁸ and is an independent predictor of decreased survival.⁹ The anemia typically worsens with disease progression and often improves during chemotherapy-induced response.¹⁰

The incidence and severity of anemia is less in the MGUS pre-malignant stage and in a more slowly progressive form of MM termed “smoldering” or “indolent” myeloma. In MGUS, anemia is rare with mean hemoglobin concentrations of >13g/dL.¹¹ In only 8 of 241 patients with MGUS was the hemoglobin less than 10g/dL and this was due to unrelated problems.¹¹ In a series of patients with smoldering/indolent myeloma, 40% of patients have normal hemoglobin levels, 60% have levels between 10 and 13 g/dL and no patients have levels below 10g/dL.¹²

Erythropoietic stimulating agents (ESAs) have been commonly used for the treatment of anemia of multiple myeloma (as well as other malignancy-associated anemias). Although numerous trials have examined the efficacy of erythropoietin derivatives in the treatment of myeloma-associated anemia, the overall impact and cost effectiveness of such treatment remains controversial.¹³ Few of these trials were powered to detect significant differences in survival. Response to ESAs is usually defined as an increase in hemoglobin of 2g/dL and response rates are consistently in the range of 60–70% (ranging from 35–85%).¹⁴ These trials demonstrated that rise in hemoglobin is due to the ESA therapy rather than change in status of the underlying myeloma. However, therapy with ESAs has been associated with increased risk of hypertension, antibody-mediated red cell aplasia and thromboembolic events.¹⁵ Targeting to a lower hemoglobin level may avoid some of these side effects. Nevertheless, as the use of ESAs has been increasingly associated with poorer survival in other malignancies,^{16–18} the current American Society of Hematology (ASH) guidelines¹⁹ for use of ESAs in myeloma advise caution: “The use of epoetin or darbopoietin may be considered for treatment-related anemia or anemia of renal failure in patients with a hemoglobin concentration < 10g/dL in order to decrease transfusion requirements. ESAs should be given at the lowest dose and frequency required to decrease transfusion requirements. Therapy should not be initiated at hemoglobin concentrations > 10g/dL and should be discontinued after 6–8 weeks if no response is seen. In rare circumstances and

only after careful consideration should ESAs be given for hemoglobin concentrations between 10 and 12g/dL.”

Several studies have addressed the pathogenesis and hematologic characteristics of myeloma-associated anemia. The anemia is usually normochromic and normocytic with evidence of hypoproliferation (reticulocyte index < 2.5%).²⁰ Iron studies demonstrate low to normal serum iron levels and elevated serum ferritin levels.²¹ Bone marrow biopsy may show an increase in hemosiderin-laden macrophages with normal to increased iron stores, consistent with impaired iron mobilization and release.²¹ As expected, patients with myeloma-associated renal disease have abnormally low levels of serum erythropoietin but even some without obvious renal impairment may have insufficient increases in erythropoietin levels for the severity of anemia.²²

There are several potential etiologies for myeloma-associated anemia that have been considered. Certainly, the extensive BM involvement with malignant cells can theoretically result in decreased capacity for functional erythropoiesis. In addition, as mentioned above, production of erythropoietin in the presence of myeloma-associated renal insufficiency is depressed and is an accepted indication for ESA treatment. An additional mechanism of anemia may be a shortened survival of RBC precursors. It has been reported that malignant plasma cells have increased expression of Fas ligand on their surface which may cause apoptosis of erythroid precursors within the marrow.²³

Although these putative pathogenic mechanisms may contribute to myeloma-associated anemia, the characteristic iron studies in patients strongly support the notion that most patients suffer from the anemia of inflammation that previously was termed “anemia of chronic disease”. It is now understood that most of these cases show impaired iron utilization due to increased pro-inflammatory cytokines that stimulate the production of the iron-regulatory hormone hepcidin. Thus, the anemia of myeloma correlates well other markers of acute phase reaction and cytokine stimulation such as ferritin levels and C-reactive protein levels. The evidence for the importance of hepcidin is summarized below.

B. Involvement of hepcidin and dysregulated iron metabolism in anemia of MM

Animal models have demonstrated that hepcidin (an acute phase reactant elevated in pro-inflammatory states) is the primary negative regulator of iron transport and release from macrophages and enterocytes.²⁴ Hepcidin, a liver-produced protein, binds to the iron exporter ferroportin, and ferroportin is then internalized and degraded.²⁵ The loss of ferroportin proportionately decreases the activity of the normal cellular pathway for iron efflux. Thus, ingested iron taken up by enterocytes cannot be released into the circulation and is lost as enterocytes are sloughed into the lumen of the GI tract. Likewise, iron catabolized from effete RBCs, obtained from hemoglobin degradation, cannot be released for re-utilization. The end result is hypoferremia, depressed iron delivery to developing RBCs, diminished hemoglobin synthesis and anemia.²⁶ Hepcidin is a potent and rapidly acting mediator of anemia: animal models have demonstrated hypoferremia within an hour of injecting hepcidin.²⁴

Hepcidin expression is induced by infection or inflammation through pro-inflammatory cytokine signaling. Cytokine activation of the JAK/STAT or SMAD signaling pathways in hepatocytes results in stimulation of the hepcidin promoter and induction of hepcidin expression as part of the type II acute phase reaction.²⁷ Interleukin-6 (IL-6) has been identified as the primary physiologic cytokine regulating hepcidin expression.²⁸ Since several studies document heightened systemic IL-6 levels in patients with multiple myeloma,^{29,30} it was tempting to hypothesize that an IL-6-induced upregulation of hepcidin expression in patients was the major cause of anemia. Thus, we studied 44 patients with

active Durie-Salmon stage III MM at diagnosis prior to therapy for whom urinary hepcidin levels could be ascertained.³¹

The mean hemoglobin concentration of this cohort was 10.1 g/dl and the RBC indices were normocytic/normochromic. The reticulocyte count was inappropriately low with all patients below 2.5%. The mean serum iron was low-normal (67 mcg/dL, normal 60–170) with normal-to-low TIBC concentrations and elevated ferritin levels (552 ng/ml, normal 22–322). These values are consistent with the anemia of chronic inflammatory disease. Urinary hepcidin levels, normalized to urinary creatinine concentrations, were three-fold higher in the myeloma patients than those of age- and sex-matched controls (n=105 healthy controls). This was statistically significant at the $p<0.0001$ level. In a smaller cohort of concurrently studied MGUS patients (n=8), who had normal hemoglobin concentrations, the mean urinary hepcidin level was not significantly different from the normal controls. Seventeen of the 44 stage III myeloma patients had some degree of azotemia (serum creatinine > 1.4). However, the mean urinary hepcidin concentration did not significantly differ between these 17 patients and the 27 with normal creatinines. Most importantly, in the 27 patients with normal renal function, the urinary hepcidin levels were highly inversely correlated with hemoglobin levels ($p=0.0014$). This inverse correlation was especially striking considering that hepcidin levels are suppressed during anemia because of increased erythropoietic drive.³² At the time of this study, a serum hepcidin assay was not yet available. A subsequent study,³³ which assayed serum hepcidin by a sensitive immunoassay,³⁴ confirmed the marked upregulation of circulating hepcidin expression in myeloma. That subsequent study³³ also found a significant inverse relationship between serum hepcidin and hemoglobin levels. These data strongly support the hypothesis that increased hepcidin is the predominant cause of anemia in patients with active symptomatic myeloma.

Further support for the etiologic role of hepcidin in the anemia of myeloma comes from sequential analysis of hepcidin levels during anti-myeloma therapy.³⁵ Eighty % of patients treated with immunomodulatory agents (IMiDs) or conventional chemotherapy had a clinical response (at least 50% fall in M-protein) and their abnormally high pre-treatment hepcidin levels significantly fell during therapy. Importantly, the hepcidin levels at approximately 1 month after starting therapy predicted the improvement of anemia in response to therapy ($p<0.05$ for increase in hemoglobin concentration of at least 2 g/dl from baseline value after 8 weeks of therapy). This was an inverse correlation (i.e., the lower the hepcidin level at this time point, the more likely the improvement in anemia). Thus, effective treatment of myeloma lowers the abnormally high serum hepcidin levels, and the lowering of hepcidin correlates with the likelihood of amelioration of anemia.

C. Mechanism of increased hepcidin production in MM

Urinary levels of hepcidin in MM patients directly correlate with serum ferritin and C reactive protein, two other proteins induced in acute phase reactions.³¹ Given that the physiologic source of hepcidin is the liver, these data suggested that cytokines stimulated hepatic hepcidin as part of the response to inflammation. As mentioned previously, the MM growth factor IL-6 was a candidate cytokine proximally stimulating hepatic synthesis of acute phase proteins in MM, including hepcidin. However, MM cells themselves rapidly and robustly respond to IL-6 with a stimulated gene expression program. To investigate if malignant cells could be the source of hepcidin, MM cells were isolated and purified from bone marrow aspirates of patients and then exposed to recombinant IL-6. There was little expression of hepcidin mRNA in these malignant primary cells and no significant increase induced by IL-6 although they responded to IL-6 with upregulated MCL-1 mRNA expression. In concurrent hepatocyte cultures, IL-6 markedly induced hepcidin mRNA (3,000 x fold higher amounts than in MM plasma cells). Thus, it is likely that the source of elevated levels of hepcidin in MM patients is the liver and not the malignant clone.

In addition to IL-6, serum levels of TNF-alpha and IL-1beta are elevated in myeloma patients^{36,37} and they both have been implicated in hepcidin regulation.^{38,39} To assess the contribution of these cytokines to elevated hepcidin in MM, in our initial cohort of 44 patients, we compared levels of urinary hepcidin with serum IL-6, TNF-alpha and IL-1 beta. There was no correlation with IL-1beta or TNF-alpha levels but the correlation between serum IL-6 and hepcidin levels was of borderline significance ($p=0.06$). Another cytokine family evaluated was bone morphogenetic proteins (BMPs), because they are potent activators of the hepcidin promoter (see below) and because prior work⁴⁰ showed high levels of BMPs in the marrow of MM patients. Serum BMP-2 levels were clearly elevated in 25 patients with active disease compared to 9 normal controls.³³ Although there was no significant correlation between BMP-2 and hepcidin levels in these sera, the relatively small sample size of this study may have precluded identification of correlations.

Because there was no clear correlation between serum or urinary hepcidin levels and specific cytokine levels (although IL-6 levels approach significance), *in vitro* examination of MM sera was undertaken. Hepcidin expression in hepatocytes is predominantly regulated at the transcriptional level and the hepcidin promoter can be stimulated via a STAT3-binding site or through BMP-responsive elements (BREs).^{41,42} The STAT3-binding site is primarily activated by IL-6 or IL-6-like cytokines transducing signals through the gp130 receptor and, subsequently, via JAK and STAT3 signaling. The BMPs activate the BREs via SMAD signaling. We evaluated the role of these pathways in the hepcidin-stimulating activity of MM sera by interfering, either at the promoter level by mutating the specific response elements or by blocking the cytokine/receptor interaction in hepatocytes.³³ A reporter assay was developed in HuH7 hepatocytes transfected with a plasmid containing the hepcidin promoter upstream of the firefly luciferase reporter gene. Sera from MM patients ($n=25$) activated hepcidin reporter expression significantly more than did normal control sera ($n=15$). There was also a significant correlation ($P=0.017$) between hepcidin concentrations in these sera and hepcidin promoter activity after stimulation of hepatocytes with the sera. Mutations in BMP-responsive elements abrogated the ability of all the sera to activate while mutations in the IL-6-responsive STAT3-binding site had less of an effect, significantly blunting the reporter expression from only 5 of 25 serum specimens.

These data supported a role for BMPs, and a lesser role for IL-6, in the hepcidin-stimulatory activity of MM sera. To further explore this notion, sera were pre-treated with either blocking antibodies to BMP-2, BMP-4, BMP-6, BMP-9, or the BMP-neutralizing protein noggin-Fc, or antibodies against IL-6 or the IL-6 receptor to neutralize IL-6 signaling.³³ These experiments identified BMP-2 as the major cytokine stimulating hepcidin promoter activity in MM sera. Blocking IL-6 signaling only prevented reporter expression from the 5 sera that had shown blunted activity when tested against the STAT-3 binding site mutant. Interestingly, recombinant IL-6 and BMP-2 were markedly synergistic in inducing hepcidin promoter activity in the hepatocyte model suggesting that, although BMP-2 was the main stimulating cytokine in MM patients, co-existing high levels of serum IL-6 could markedly enhance hepcidin expression within the livers of myeloma patients.

These results collectively support the model depicted in Figure 1: The growth of the MM clone within the bone marrow stimulates paracrine expression of BMP-2 and IL-6. The resulting elevated serum levels of these cytokines activate the hepcidin promoter in hepatocytes. Most promoter activation is due to BMP-2 signaling through SMADs to the BREs but IL-6 can also signal the promoter at its STAT3 binding site via the gp130/JAK/STAT pathway with a synergistic interaction at the level of the hepcidin promoter to further enhance hepcidin expression. Hepatocytes secrete increased amounts of hepcidin which then circulates and binds to ferroportin on enterocytes and macrophages, resulting in degradation of ferroportin. Iron is trapped in enterocytes or erythrocyte-recycling macrophages, no

longer replenishing the small iron compartment in plasma, which then is rapidly depleted and hypoferrremia ensues. The diminished supply of iron limits hemoglobin synthesis leading to anemia. When anti-MM therapy is successful, IL-6 and BMP-2 levels fall, hepcidin expression is down-regulated, iron supply to erythropoietic cells increases and anemia abates. The model makes several predictions. First, at moderate doses, erythropoietin is not likely to have a marked success in patients because impaired delivery of iron will be the limiting barrier to erythropoiesis. The ability of higher doses of erythropoietin to suppress hepcidin could overcome this barrier, at the expense of dose-related side effects. Second, addition of iron to erythropoietin is also not likely to be very successful because administered iron taken up by enterocytes (after oral ingestion) or by macrophages after an initial modest incorporation into RBCs from parenteral administration, will continually be locked in these cells with no egress unless hepcidin levels are lowered; and third, anti-IL-6 antibody therapy might not by itself have a major effect on hepcidin levels or anemia if there is no concurrent down-regulation of BMP-2 expression.

III. THERAPEUTIC INDUCTION OF IRON DEPRIVATION FOR THE TREATMENT OF MYELOMA

A. Iron entry into cancer cells

Iron is an essential metal for life and is required for many cellular processes including DNA synthesis, oxygen transport, and cell growth.^{43,44} However, iron can also be toxic due to its facilitation of oxygen radical production. To limit toxicity of the free element, iron travels through the blood bound to a protein called transferrin (Tf). Cellular uptake of iron occurs through the interaction of Tf with its receptor, the transferrin receptor (TfR).⁴⁴ After binding its receptor, iron-loaded Tf is internalized by receptor mediated endocytosis (Figure 2). There are two receptors that can mediate iron import, TfR1 (also known as CD71) and TfR2.⁴⁴ The TfR1 has a much higher affinity for Tf compared to TfR2. In general, the TfR1 is ubiquitously expressed at low levels on most cells, while the expression of TfR2 is largely restricted to hepatocytes. However, TfR1 is overexpressed on cells with a high rate of proliferation including many types of cancer cells including malignant hematopoietic cells. Within the cell, iron is a required co-factor for the ribonucleotide reductase, an enzyme necessary for the conversion of ribonucleotides into deoxyribonucleotides that is essential for DNA synthesis and is also often overexpressed in cancer cells.^{43,44} Without iron this enzyme is rendered inactive leading to cell cycle arrest. Due to the high rate of proliferation and increased metabolism, cancer cells have an increased need for iron making them more susceptible to the disruption of iron metabolism.^{43,45} Thus, direct iron chelation or blockage of iron uptake through the TfR have both been explored as potential cancer therapies. Our discussion here is focused on compounds that interfere with iron metabolism and are promising therapeutic approaches against the hematopoietic malignancy multiple myeloma (MM).

B. Direct iron chelation as a therapeutic option

One way to disrupt iron metabolism in cancer cells is the direct chelation of iron to deplete intracellular iron levels. The iron chelator desferrioxamine (DFO) produced by the bacterium *Streptomyces pilosus* has been used for the treatment of iron overload disease and has also shown anti-cancer effects.^{43,45} However, the utility of DFO is limited due to its poor membrane permeability and short plasma half-life.^{43,45} Many DFO analogs have been prepared in order to overcome these problems. One of these, the synthetic iron chelator di-2-pyridylketone-4,4-dimethyl-3-thiosemicarbazone (Dp44mT), has been shown to have anti-cancer activity in a wide variety of cancer cells without causing iron-depletion through the body.^{46,47} The U266 and RPMI 8226 human multiple myeloma (MM) cell lines are highly sensitive to the cytotoxic effects of Dp44mT *in vitro*,⁴⁸ while the chelator had very limited

effects on normal peripheral blood mononuclear cells (PBMC), thus demonstrating a possible therapeutic window. Dp44mT has increased anti-tumor activity when compared head to head with DFO. This has been attributed to its superior lipophilicity.⁴⁸

Another potential myeloma drug that could cause iron deprivation is gallium. Gallium is a group IIIA metal with similar properties to those of iron.⁴⁹ Gallium also binds Tf in the blood⁵⁰ and the Tf-gallium complex can bind the transferrin receptor and is internalized into cells by receptor-mediated endocytosis. Iron uptake into tumor cells can be blocked by the gallium-Tf complex or by a prevention of acidification of Fe-Tf-containing endosomes (which would inhibit release of iron into the cytosol⁴⁹). Additionally, gallium can interfere with cell metabolism through the direct inhibition of the ribonucleotide reductase enzyme.⁴⁹ It is interesting that gallium has demonstrated some success in myeloma patients as a therapeutic. Although its rationale initially was as an agent preventing myeloma bone destruction and a phase I clinical trial⁵¹ supports that role, a second trial⁵² showed that patients with MM treated with the M-2 chemotherapy regime (vincristine, carmustine, cyclophosphamide, melphalan, and prednisone) plus gallium nitrate had markedly prolonged median survival compared to patients treated with chemotherapy alone. Although there are several other possible explanations for the gallium-dependent prolongation of survival, this study begs the question of whether gallium induced an additional anti-tumor effect via intracellular iron deprivation.

Other compounds have been shown to bind intracellular iron leading to toxic effects. The antimicrobial agent cicopirox olamine (CPX), which is currently approved for the treatment of cutaneous fungal infections, has been evaluated for its iron-chelation properties and anti-cancer effects.⁵³ CPX was shown to bind intracellular iron that resulted in the decrease of viability and cell growth of 8 human MM cell lines *in vitro*, including KMS-11 and OCI-My5. This cytotoxicity was shown to be dependent on iron since the additional of excess iron blocked this effect. Additionally, CPX analogs that could not bind iron did not exhibit this cytotoxicity. CPX is an effective anti-cancer agent in murine models as well.⁵³

An additional potential disruptor of MM cell iron metabolism is curcumin. Clinical trials are currently evaluating curcumin in MM patients after some promising pre-clinical studies.⁵⁴ Curcumin is a polyphenolic extract isolated from the spice turmeric (*Curcuma longa*). This plant extract is lipophilic, readily permeates cell membranes, and has been shown to have many beneficial properties including anti-inflammatory, antioxidant, and chemotherapeutic activity due to its complex structure and its ability to influence multiple cell signaling pathways.⁵⁵ It can also bind iron and has been shown to be an iron chelator.^{55,56} Studies in humans have shown that curcumin is remarkably well tolerated even at doses as high as 8 grams per day.⁵⁵ A Phase I clinical trial has been conducted in patients with MM (ClinicalTrials.gov Identifier NCT00113841) where curcumin was given orally twice daily at 2 grams per dose. The effects of curcumin alone or combined with Bioperine, a pepper extract that increases the absorption of nutrient supplements, were evaluated. No serious adverse events were reported in either group. The primary endpoint was NF- κ B protein expression in PBMC compared to baseline levels. Protein expression did not increase in either treatment group. A Phase II clinical trial (ClinicalTrials.gov Identifier NCT01269203) is in progress and opens for recruitment in October 2012. This trial will be conducted in MM patients also being treated with lenalidomide (versus placebo) as a maintenance therapy. The goal of this trial will be to determine if curcumin can reduce the symptoms of MM during maintenance therapy.

C. Iron deprivation induced through the use of antibodies targeting the transferrin receptor

Blocking iron metabolism can also be accomplished through the use of antibodies targeting the TfR1.⁴⁴ This strategy exploits the over-expression of the receptor in cancer cells and its

central role in cancer cell pathology. Antibodies specific for the TfR1 that are directly cytotoxic to the cell through the induction of iron starvation can interfere with iron uptake in two ways. They can be neutralizing antibodies in that they inhibit the binding of Tf to the receptor and thus block iron uptake and/or they can be non-neutralizing antibodies that still allow Tf to bind the receptor, but induce iron deprivation by disrupting the normal cycling pathway of TfR1. Antibodies have additional anti-tumor mechanisms through their effector functions including antibody-dependent cell-mediated cytotoxicity (ADCC), antibody-dependent cell-mediated phagocytosis (ADCP), and complement-mediated cytotoxicity (CDC). In fact, the mouse/human chimeric antibody D2C specific for the TfR was shown to induce ADCC activity against human cancer cells.⁵⁷ Furthermore, antibodies targeting the TfR1 have the added benefit of being able to act as delivery vehicles to internalize anti-cancer agents by receptor-mediated endocytosis, which can also potentially trigger a cytotoxic effect even if the antibodies do not have a direct anti-cancer effect.^{58,59}

Various antibodies targeting the TfR have shown anti-cancer effects against myeloma cells. A rat anti-murine TfR IgM antibody (R17 208) blocks TfR internalization and iron uptake causing growth inhibition and G2/M arrest in S194/5.XXO.BU.1 murine MM cells.⁶⁰ Another antibody, the murine IgA anti-human TfR antibody 42/6 is cytotoxic to RPMI 8226 human MM cells *in vitro*.⁶¹ This antibody inhibits binding of Tf to its receptor and additionally induces down-regulation of the receptor on the cell surface.⁶² The 42/6 therapeutic has been studied in a prior phase I clinical trial.⁶³ Additional murine anti-human TfR IgG1 antibodies (E2.3 and A27.15) have also shown cytotoxicity against 8226 and OCI-MY4 human MM cells *in vitro*.⁶⁴

Two antibody avidin fusion proteins targeting the TfR have also been studied for their anti-myeloma effects. One of these fusion proteins (ch128.1Av; formerly known as anti-hTfR IgG3-Av) contains the variable regions of the murine antibody 128.1 and targets the human TfR, while the second (anti-rat TfR IgG3-Av) targets the rat TfR and contains the variable regions of the murine antibody OX26.⁶⁵ Both of these fusion proteins consist of mouse/human chimeric IgG3 genetically fused to chicken avidin at the C_H3 domains of each heavy chain. These molecules were designed to be universal vectors that can deliver a wide variety of biotinylated therapeutic agents into cancer cells by receptor-mediated endocytosis. Additionally, both fusion proteins have been shown to exhibit superior intrinsic cytotoxic activity compared to their parental antibody without avidin against a variety of malignant hematopoietic cell lines, including myeloma cells of their respective species.⁶⁵⁻⁶⁷ Ch128.1Av was also cytotoxic to primary cells isolated from MM patients including a case of plasma cell leukemia (PCL), the most aggressive presentation of MM.⁶⁶ Ch128.1Av significantly alters the classical recycling pathway of the TfR, redirecting it to lysosomal compartments, the site in which it is presumably degraded. As a result, the surface level of the TfR is dramatically reduced leading to lethal iron deprivation characterized by mitochondrial depolarization and activation of caspases 2, 9, 8, and 3.⁶⁶⁻⁶⁹

The *in vivo* anti-myeloma effect of ch128.1Av has been evaluated in two disseminated human MM models in immunodeficient mice.⁷⁰ For these two models either ARH-77 or KMS-11 cell lines were injected intravenously (i.v.) resulting in disseminated spread. A single dose of ch128.1Av resulted in significant anti-tumor activity by prolonging the survival of treated animals in both of these xenograft models.⁷⁰ The results using KMS-11 were surprising given the limited *in vitro* sensitivity to this fusion protein. In addition, the parental antibody without avidin (ch128.1) also showed remarkable *in vivo* anti-cancer activity under the same conditions despite its limited *in vitro* direct cytotoxicity against both ARH-77 and KMS-11 cells. This suggests that the iron starvation is exacerbated *in vivo* and/or the antibody effector functions are involved in the anti-tumor activity observed in the animal. Interestingly, the *in vivo* ch128.1 anti-tumor activity was superior compared to a

similar dose of ch128.1Av. This superior protection of ch128.1 can be explained by a lower bioavailability of ch128.1Av, which contains avidin and, thus, it is expected to clear faster from the blood.⁷⁰ In fact, in studies conducted in a one-week time frame in mice it was observed that ch128.1Av clears from the blood much faster compared to ch128.1. While ch128.1Av cannot be detected in the blood 24 hours after injection, more than half of the injected dose of ch128.1 can be detected in the blood of animals at this period. The significant anti-tumor activity of ch128.1Av, despite its limited bioavailability, suggests that this molecule is highly effective as an anti-tumor agent and that at higher doses and/or multiple administrations, it might be possible to achieve an anti-tumor effect that cannot be matched by the parental antibody. This protective effect may also be increased by conjugating ch128.1Av to biotinylated drugs. It is important to emphasize that a short half-life may be desirable in certain applications and conditions to limit unwanted side effects. Taken together, these results suggest that ch128.1 and ch128.1Av have a potential as treatments for MM.

Since ch128.1Av is cytotoxic to malignant hematopoietic cells, its toxicity in normal human hematopoietic stem (HSC) and early progenitor cells was evaluated using the long-term cell-initiating culture (LTC-IC) assay.⁷¹ This *in vitro* assay enumerates the number of pluripotent stem/non-committed progenitor cells and is used to evaluate the toxicity of potential therapeutics against HSC. ch128.1Av is not toxic to HSC cells *in vitro* under conditions in which the ch128.1Av is toxic to malignant B-cells. These results are consistent with the reported lack of expression of TfR in HSC^{71–73} and suggest that this relevant cell population, capable of both self-renewal and generation of lineage-restricted progenitors, would be preserved in patients treated with ch128.1Av or ch128.1. However, effects on more mature, committed erythropoietic cells might be problematic, especially since these cells in myeloma patients are operating at already deficient levels of available iron as discussed in the first section of this review.

SUMMARY

The above studies demonstrate the importance of iron metabolism in multiple myeloma patients. First, it is very clear that the almost universal occurrence of anemia in myeloma patients is due to hypoferremia and decreased availability of iron for the developing erythrocyte. This reduced iron availability results from a cascade of events initiated by upregulated expression of the cytokines BMP-2 and IL-6 in patients. High levels of circulating cytokines then stimulate hepatocytes for secretion of the acute phase reactant, hepcidin. Hepcidin then binds to ferroportin on enterocytes and cells of the RE system, preventing iron egress with resulting hypoferremia. Theoretically, future therapeutics that could target hepcidin (or possibly BMP-2) would ameliorate the anemia of myeloma.

Secondly, it is clear that the myeloma tumor cell has a heightened requirement for iron to support its proliferation and viability. There are a number of therapeutics targeting iron availability for the myeloma cell that have shown potential in pre-clinical studies. The major hurdle for their continuing development will be difficulty in demonstrating tumor cell-specificity since iron is also important in rapidly proliferating non-malignant cells.

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Abbreviations

MM	multiple myeloma
MGUS	monoclonal gammopathy of undetermined significance
ESAs	erythropoietic stimulating agents
ASH	American Society of Hematology
BM	bone marrow
RBC	red blood cell
IMiDs	immunomodulatory agents
BMPs	bone morphogenetic proteins
BRE	BMP-responsive element
Tf	transferrin
TfR	transferrin receptor
DFO	desferrioxamine
PBMC	peripheral blood mononuclear cell
CPX	cicopiroxolamine
ADCC	antibody-dependent cell cytotoxicity
CDC	complement dependent cytotoxicity

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Figure 1.

Model of pathogenesis of anemia in multiple myeloma. The malignant myeloma clone stimulates production of BMP-2 and IL-6 in non-malignant cells of the marrow microenvironment (such as stromal cells, bone cells and endothelial cells). These cytokines are released into the systemic circulation, reach the liver and then stimulate hepatocyte signal transduction which results in activation of the hepcidin promoter, hepcidin transcription and protein expression. Hepcidin levels are increased systemically and this hormone acts on its receptor/iron exporter ferroportin to block the efflux of iron from the reticuloendothelial system and from enterocytes. Plasma iron levels are then greatly diminished and iron-restrictive anemia ensues.

Figure 2.

Cellular uptake of iron through the Tf system via receptor-mediated endocytosis. Iron-loaded Tf (diferric-Tf or holo-Tf) interacts with the TfR and endocytosis occurs via clathrin-coated pits where the complex is delivered into endosomes. Protons are pumped into the endosome resulting in a decrease in pH that stimulates a conformational change in Tf and its subsequent release of iron. The iron is then transported out of the endosome into the cytosol by the divalent metal transporter 1 (DMT1). Apo-Tf (devoid of iron) remains bound to the TfR while in the endosome and is only released once the complex reaches the cell surface. Reprinted from Daniels et al. Clin. Immunol. 2006 Nov;121(2):144–58 with permission from Elsevier.